DISTRIBUTION OF METABOLITES BETWEEN MITOCHONDRIA AND CYTOSOL OF CULTURED FIBROBLASTOID RAT HEART CELLS

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1. Introduction

The energy metabolism of the cell is compartmented between the mitochondrial and cytosolic space. The adenine nucleotide translocator situated in the inner mitochondrial membrane [1] enables a communication between the ATP systems in the two subcellular compartments. For a study of the ATP-generating and -consuming processes the measurements of subcellular adenine nucleotide contents are required.

Recently, a method for the assay of subcellular metabolite contents in perfused tissues has been described [2]. This method involves a freeze fixation of the tissue, lyophilisation of the frozen material and homogenisation and fractionation of the dried tissue in non-aqueous solvents. Here, this method has been adapted for the analysis of subcellular contents of ATP, ADP, P_i, creatine phosphate and creatine in cultured fibroblastoid rat heart cells.

2. Materials and methods

2.1. *Cultivation of fibroblastoid rat heart cells*

A cell suspension has been obtained by trypsinisation of hearts from day 1–5 rats [3] consisting of fibroblastoid cells and muscle cells. The fibroblastoid cells were separated as in [4] and subsequently cultivated in 175 cm^2 flasks (Nunc) in a medium [5] CMRL 1415 without bicarbonate, supplemented with 10% fetal calf serum and adjusted to pH 7.4 (medium and serum were purchased from Seromed, München). After reaching a saturation density of $\sim 10^5$ cells/cm^2, the cells were subcultivated (0.25% trypsin from Serva, Heidelberg; 3×10^4 cells/cm^2) and this procedure repeated 1–3 times.

2.2. *Freeze fixation and fractionation in non-aqueous solvents*

The cells were washed twice with a solution containing 135.0 mM NaCl, 4.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.0 mM D-glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (Hepes) adjusted to pH 7.4 with NaOH, at 37°C. The cell monolayer covered with a thin film of adherent fluid and thus well aerated was then freeze fixated by pouring liquid nitrogen into the flasks. The cells still contained in the flasks were then lyophilised at –60°C and 0.001 T. Flasks (10–40) with a total amount of 50–200 mg protein were cut open with a heated steel scraper. The cells were scraped off in a desiccated glove box and suspended in 11 ml heptane–CCl_4, $d = 1.38$ g $\times$ cm^{-3}, 4°C and sonicated periodically with a sonifier 'Branson', type B 15, for 5 s, followed by an interval of 5 s for a total of 4 min. During the sonification the suspension was cooled in a mixture of heptane and dry ice. The homogenate was then fractionated by density gradient centrifugation into 6–8 fractions (detailed in [2]).

2.3. *Assays*

Marker enzymes for the mitochondrial matrix (citrate synthase [6]) and cytosolic compartments (phosphoglycerate kinase [7]), protein [8], ATP, ADP, creatine, creatine phosphate and P_i [6,9] were

determined in each fraction obtained from the density gradient centrifugation.

2.4. *Calculations*

Mitochondrial and cytosolic contents of ATP, ADP, creatine, creatine phosphate and P_i (nmol/mg protein) were obtained by extrapolating the values of the fractions of the density gradient to fractions of pure mitochondria and cytosol according to [2].

2.5. *Chemicals*

Chemicals were purchased from Merck (Darmstadt) Boehringer (Mannheim) and Sigma (München).

3. Results and discussion

3.1. *Subcellular metabolite contents in fibroblastoid rat heart cells*

Table 1 (A) shows the subcellular distribution of protein, ADP and ATP obtained with 7 different cell preparations cultured aerobically under standard conditions (see section 2). In some of the experiments P_i, creatine phosphate and creatine were also measured. The amount of cell material available was too low for an accurate determination of AMP.

It appears from our measurements that ~20% of the total protein belongs to the mitochondria. Compared to rat hepatocytes, in which mitochondria represent ~35% of the total protein [2], the fibroblastoid cells have a low content of mitochondria. The total amount of ATP and ADP as related to mitochondrial and extramitochondrial protein, respectively, is very similar to that found in perfused liver [10]. Also in fibroblastoid cells the extramitochondrial ATP/ADP ratio is higher than the mitochondrial ratio, though the differences are not as large as in perfused liver [10]. Apparently also in fibroblastoid cells the export of ATP from the mitochondria which is mediated by the adenine nucleotide translocator, is an energy requiring process. The amount of inorganic phosphate in the cytosol, as related to the extramitochondrial protein, is found to be ~15-times higher than the mitochondrial phosphate content (expressed as nmol/mg mitochondrial protein). Since the subcellular water content of fibroblastoid cells has not been determined, we are at present unable to express our results in terms of concentrations. In perfused liver the subcellular water spaces were estimated to be 3.8 μl/mg extramitochondrial protein and 0.8 μl/mg mitochondrial protein [11]. Assuming that these values are also relevant for fibroblastoid

Table 1
Subcellular content of metabolites in fibroblastoid rat heart cells

	Total cells (nmol/mg tot. protein)		Cytosol (nmol/mg cyt. protein)		Mitochondria (nmol/mg mitochondrial protein)		% total content	
	A	B	A	B	A	B	A	B
ATP	17.8 ± 1.1	2.9	24.6 ± 2.5	2.7	5.0 ± 0.8	3.8	5	11
ADP	3.6 ± 0.4	3.8	4.4 ± 0.6	3.6	2.2 ± 0.3	5.3	11	8
P_i	5.0 (3.5–6.9)	–	6.5 (5.4– 8.5)	–	1.3 (0.9–2.0)	–	5	–
Creatine	2.9 (2.2–3.3)	4.9	3.6 (2.6– 4.3)	5.6	0.3 (0.1–0.5)	1.6	2	7
Creatine phosphate	6.7 (5.2–7.7)	0.4	8.8 (6.7–10.9)	–	0.1 (0.1)	–	0	–
protein							21	18
ATP/ADP	4.9	0.76	5.6	0.75	2.3	0.72		
creatine phosphate/ creatine	2.9	0.1	2.4	–				

Expt. A: Standard incubation (see section 2); the data represent mean values. ATP, ADP and protein measurements: $n = 7$ (± SEM). Other measurements $n = 3$ (maximal–minimal values). Expt. B: Data represent a single experiment (reproduced by others): in this experiment glucose was omitted from the washing medium and the cells were incubated for 20 min with 1 μM FCCP in buffered salt solution without glucose (see section 2.2) prior to the freeze stop. Under condition A ~90% of the cells excluded trypan blue, whereas under condition B 20% of the cells excluded trypan blue

rat heart cells, the data of table 1 would indicate that the phosphate concentrations in both compartments are about equal. This would concur with the distribution of P_i in perfused liver [10,11].

As shown in table 1, creatine phosphate is located in the extramitochondrial compartment exclusively. This result was to be expected since the inner mitochondrial membrane is impermeable to all phosphate containing compounds except those being transported by specific carriers. Such a specific transport of creatine phosphate may be regarded as most unlikely, since creatine kinase was found to be located in the cytosol and in the mitochondrial intermembrane space [12,13].

It has been also observed in isolated mitochondria that creatine phosphate is unable to permeate into the matrix space [14]. The absence of creatine phosphate in the matrix space, as found in our experiments may therefore be regarded as evidence for the reliability of the non-aqueous fractionation procedure.

In contrast to creatine phosphate, a considerable amount of creatine is found also in the mitochondrial compartment. It appears that the inner mitochondrial membrane is not completely impermeable for creatine. This concurs with earlier results obtained with isolated mitochondria [14].

3.2. *The effect of uncoupler on the subcellular distribution of ATP and ADP*

Table 1 also shows the effect of the uncoupler FCCP (carbonylcyanid-*p*-trifluoromethoxyphenylhydrazone) on the subcellular distribution of ATP and ADP and the subcellular adenosine nucleotide contents. As compared to the control values, a decrease of the overall ATP/ADP (and creatinephosphate/creatine) ratios was observed. The breakdown of creatinephosphate leads to a considerable increase of the creatine content not only in the cytosol but also in the mitochondrial compartment. Especially in the cytosol, the sum of ATP + ADP is much lower. Provided that an adenylate kinase-mediated equilibrium exists in the cytosol, as found in perfused liver [10], the decrease of the sum of ATP + ADP would also reflect a decrease of the sum of ATP + ADP + AMP by ~50% compared with the control. A similar decrease of ATP and ADP on the addition of uncoupler has been observed with isolated hepatocytes [15]. Since it was found, that ~80% of the fibroblastoid cells were permeable to trypan blue after incubation with FCCP for 20 min (see table 1 legend), this decrease may be due to loss of metabolites into the medium. Furthermore it may reflect a breakdown of accumulated AMP.

The difference between the mitochondrial and extramitochondrial ATP/ADP ratios disappeared after the addition of uncoupler. It has been shown earlier with isolated mitochondria [16] and also in perfused liver [10] that uncoupling of electron transport abolishes the specificity of the adenine nucleotide translocator for ATP export out of the mitochondria into the cytosol against a potential gradient. It appears from the data in table 1 that this is also the case in fibroblastoid rat heart cells.

3.3. *Concluding remarks*

Our results show that the non-aqueous fractionation procedure for measurement of subcellular metabolite contents can be not only applied to perfused organs but also to isolated and/or cultivated cells. This method might therefore also enable the study of the subcellular metabolism in different cell types of an organ, e.g., muscle cells and fibroblastoid cells of myocardium. Thus, a more detailed analysis of the contribution of each cell type to the metabolism of the whole organ might be possible. Furthermore, working with cell cultures will allow the study of subcellular metabolism in defined cell strains and lines, e.g., tumor cells and genetic variants.

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